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Lirula sichuanensis sp. nov. on *Picea likiangensis* var. *rubescens* from Sichuan, China

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Abstract—*Lirula sichuanensis* on 2-year-old dead needles attached on living twigs of *Picea likiangensis* var. *rubescens* [= *P. likiangensis* var. *balfouriana*] in Sichuan Province is described and illustrated as a new species. Phylogenetic analysis based on the internal transcribed spacer sequences of ribosomal DNA sequences confirms its distinction from similar species of the genus. The type specimen is deposited in the Forest Protection Laboratory of Sichuan Agricultural University, China (SAUF).

Key words—*Rhytismataceae*, needle blight, taxonomy, ITS rDNA

Introduction

Lirula Darker is distinguished from other genera of *Rhytismataceae* by its nervisequious ascomata and ascospore shape (Darker 1967). Initially, six species were recombined in *Lirula* by Darker (1967): *L. abietis-concoloris* (Mayr) Darker, *L. macrospora* (R. Hartig) Darker, *L. mirabilis* (Darker) Darker, *L. nervata* (Darker) Darker, *L. nervisequa* (DC.) Darker, and *L. punctata* (Darker) Darker. Ziller (1969) described a new species, *L. brevispora* Ziller on *Picea glauca* (Moench) Voss from Canada. Kaneko (1993) described *L. pakistanensis* S. Kaneko on *Abies pindrow* (Royle ex D. Don) Royle from Pakistan and later (Kaneko 2003) described *L. exigua* S. Kaneko and *L. japonica*

S. Kaneko on *Abies mariesii* Mast. from Japan. Fan et al. (2012) described *L. yunnanensis* L. Fan et al. on *Abies georgei* Orr [≡ *A. forrestii* var. *georgei* (Orr) Farjon] from Yunnan Province, China.

The eleven *Lirula* species usually cause severe needle blight on conifers; two species parasitize *Picea*, and the others mainly *Abies* (Table 1). In this paper, we report a new species of *Lirula* on dead needles of *Picea likiangensis* var. *rubescens* [= *P. likiangensis* var. *balfouriana* (Rehder & E.H. Wilson) Hillier].

Materials & methods

Mature fruitbodies were selected and examined macroscopically under a dissecting microscope at 6–45× magnification and microscopically under a compound microscope at 40–100× magnification (objective lens). The fruitbodies were sectioned by hand; vertical sections were mounted in water for observation of ascomatal and conidiomatal outlines. Gelatinous sheaths surrounding ascospores and paraphyses were examined in water or cotton blue lactophenol. The colours of anatomical structures and ascospore contents were observed in water. All measurements were made using material mounted in water.

Total genomic DNA was extracted from ascomata following the operations manual of DNA extraction kit™ (Tiangen, China). The internal transcribed spacer sequences of ribosomal DNA (ITS rDNA) region were amplified with PCR using the primers ITS1/ITS4 (White et al. 1990). PCR was performed in 50-µL reactions including DNA template 5 µL, primer ITS1/ITS4 2 µL, 2× MasterMix 25 µL, and ddH_2O 16 µL, under the following parameters: 95 °C for 1 min., 50 °C for 1 min., 72 °C for 1 min., for a total

Table 1. Hosts of *Lirula* species.

Species	Host	Reference
L. abietis-concoloris	*Abies concolor*	Darker 1967; Scharpf 1988
L. brevispora	*Picea glauca*	Ziller 1969
L. exigua	*Abies mariesii*, *A. georgei*	Kaneko 2003; Fan et al. 2012
L. japonica	*Abies mariesii*	Kaneko 2003; Fan et al. 2012
L. macrospora	*Picea likiangensis* var. *rubescens, P. wilsonii*, *Picea asperata*, *P. crassifolia*, *P. jezoensis, P. koraiensis*, *P. neoveitchii*, *P. schrenkiana*, *P. abies*, *P. glauca*, *P. pungens*	Darker 1932; Darker 1967; Lin et al. 2012; Scharpf 1988
L. mirabilis	*Abies balsamea*	Darker 1967
L. nervata	*Abies balsamea*	Darker 1967
L. nervisequa	*Abies alba*, *A. fabri*, *A. fargesii*, *A. holophylla*, *A. forrestii* var. *smithii*, *Taxus wallichiana*	Darker 1967; Lin et al. 2012; Fan et al. 2012
L. pakistanensis	*Abies pindrow*	Kaneko 1993
L. punctata	*Abies amabilis, A. balsamea, A. concolor, A. grandis, A. procera, A. mariesii*	Darker 1967
L. yunnanensis	*Abies georgei*	Fan et al. 2012

of 35 cycles followed by a final extension step at 72 °C for 5 min. The PCR products detected by gel electrophoresis were sent to Invitrogen Biotechnology (Guangzhou, China) for purification and sequencing.

The ITS rDNA sequences were aligned with ClustalW 1.8 (Thompson et al. 1997); sections of the sequences longer than the sequence of new species were excluded from the analysis. A neighbor-joining (NJ) tree was generated with MEGA5.1 software (Tamura et al. 2011), and branch stability was estimated with 1000 bootstrap pseudo-replicates calculated with Kimura's two-parameter model (Kimura 1980). Other ITS rDNA sequences used in this study were downloaded from GenBank (Table 2). The newly generated sequences were deposited in GenBank.

Table 2. ITS rDNA sequences of the species used in the study and their GenBank accession numbers. The holotype of *L. sichuanensis* is indicated as [HT].

Species	Voucher	GenBank No.	Reference
*Lirula macrospo*ra		AF462440	NCBI
		AF462441	NCBI
Lirula yunnanensis		HQ902156	Fan et al. 2012
Lirula exigua		HQ902157	Fan et al. 2012
		HQ902154	Fan et al. 2012
		HQ902155	Fan et al. 2012
Lirula sichuanensis	SAUF1605001 [**HT**]	KY399845	This paper
	SAUF1606001	KY399846	This paper
*Lophodermium coni*genum		AY422489	Pan et al. 2006
*Lophodermium austr*ale		KM106810	Oono et al. 2014
		KM106812	Oono et al. 2014
*Lophodermium ind*ianum		KF636510	NCBI
		KF636502	NCBI
*Rhytisma panamens*e		GQ253102	Hou et al. 2010
*Rhizosphaera kalkhoff*ii		KM435342	Azeem et al. 2015
		JQ353721	You et al. 2013

Taxonomy

Lirula sichuanensis X.L. Xu, C.L. Yang & Y.G. Liu, **sp. nov.** Figs 1–12

MycoBank MB 819592

Differs from *Lirula macrospora* by its larger asci and ascospores, its branched, unswollen (or only slightly swollen) paraphyses, and its hypophyllous conidiomata forming a continuous or interrupted row on both sides of the substrate needle's stomatal line.

Type: China, Sichuan Province, Pingwu County, Yangdonghe Plantation, alt. 2822 m, on needle of *Picea likiangensis* var. *rubescens* Rehder & E.H. Wilson (*Pinaceae*), 5 May 2016, X.L. Xu & C.L. Yang 160504 (**Holotype**, SAUF1605001; GenBank KY399845).

Etymology: *sichuanensis*, referring to Sichuan Province where the specimens were collected.

Ascomata hypophyllous (occasionally epiphyllous), nervisequious, on previous year dead needles but still attached to a living twig; dull to shining black in surface view, (2–)4–12(–17) × 0.38–0.6 mm, no perimeter line, variably long and extending at times almost the entire length of the needle; intra-epidermal in median vertical section, 190–330 µm deep; strongly raised above the surface of the substrate, opening widely by a single longitudinal split, exposing the canary yellow hymenium. Covering stroma conspicuous and well carbonized, no lip cell observed; ≤96–133 µm thick near the ascomal centre, becoming thinner towards the edges and not extending to the basal layer; consisting of an outer layer of host cuticle and epidermal cell remnants, an inner layer of brown to black brown textura angularis with 6–11 µm diam. cells, and an innermost layer of colourless to pale brown textura globulosa with 7–12 µm diam. cells. Hymenium concave to flat, (80–)100–210 µm thick. Subhymenium hyaline, deep concave, composed of hyaline, pseudoparenchymatous cells. Basal stroma poorly developed or almost absent. Paraphyses filiform, straight or curved, longer than asci, 180–240 µm long, 2.6–4.2 µm thick at the base, multi-septate, tapering towards the tip, 1.4–2.3 µm thick, not or only slightly swollen, often branched near the top or in the middle, surrounded by a gelatinous matrix 1.7–2.7 µm thick, but not forming an epithecium. Asci clavate, subtruncate at the apex when maturing, tapered below to a short stipe, 105–166 × 16–23 µm, J–, 8-spored. Ascospores cylindrical-clavate, hyaline, arranged in fascicles, 51–90 × 5–7 µm, rounded at the apex, tapered towards the base, encased in a conspicuous (3.7–6.6 µm thick) hyaline gelatinous sheath.

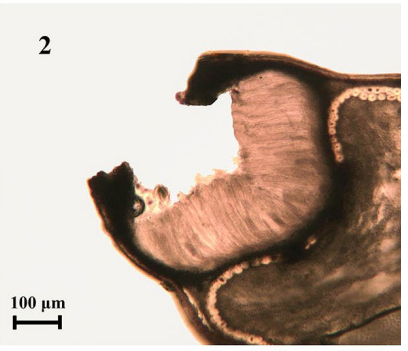
Conidiomata hypophyllous, on both sides of the stomatal lines, forming two continuous or interrupted rows on dead needles attached to living twigs, 110–360 µm wide, intra-epidermal in vertical section, 58–65 µm deep; concolorous with the substrate or pale brown, appearing as shining blisters, round or ellipse or irregular shape, opening by sparse little ostioles. Upper wall absent. Basal wall extremely poorly developed. Conidia hyaline, baculiform, straight or slightly curved, 4–7 × 2 µm.

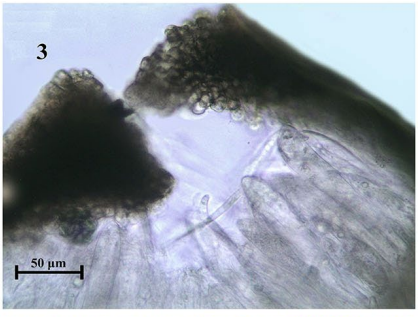
Zone lines not observed.

Habitat & distribution: Producing conidiomata and ascomata on dead needles still attached to living twigs. Known only from Sichuan Province, China.

Figs. 1–8. *Lirula sichuanensis* (holotype, SAUF1605001) sexual stage on *Picea* needles. 1. Ascomata observed under the dissecting microscope. 2. Ascoma in vertical section. 3. Covering stroma near the centre of ascoma in vertical section. 4. Asci and paraphyses. 5. Paraphysis bases and a young ascus. 6. Paraphysis tips. 7. Ascus containing eight ascospores. 8. A single released ascospore.

1
1 mm


2
100 µm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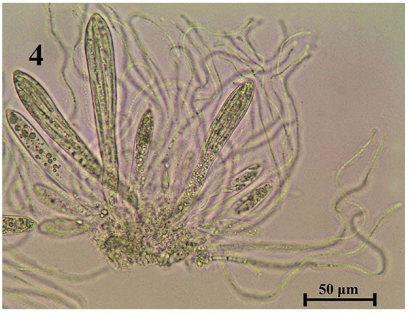
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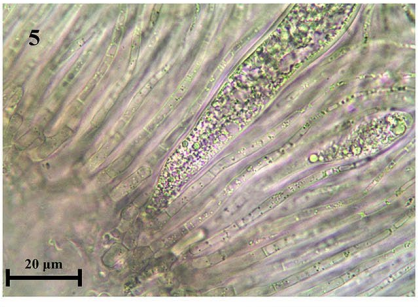
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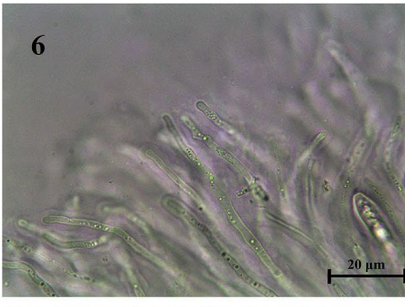
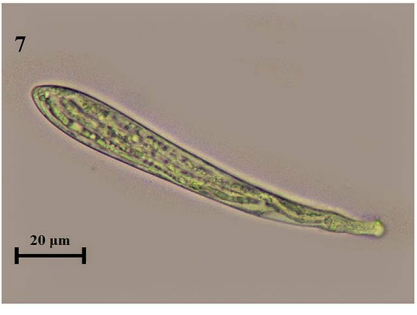

6
20 µm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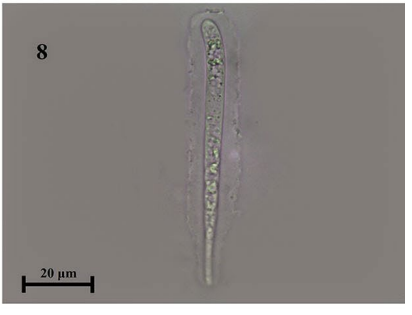
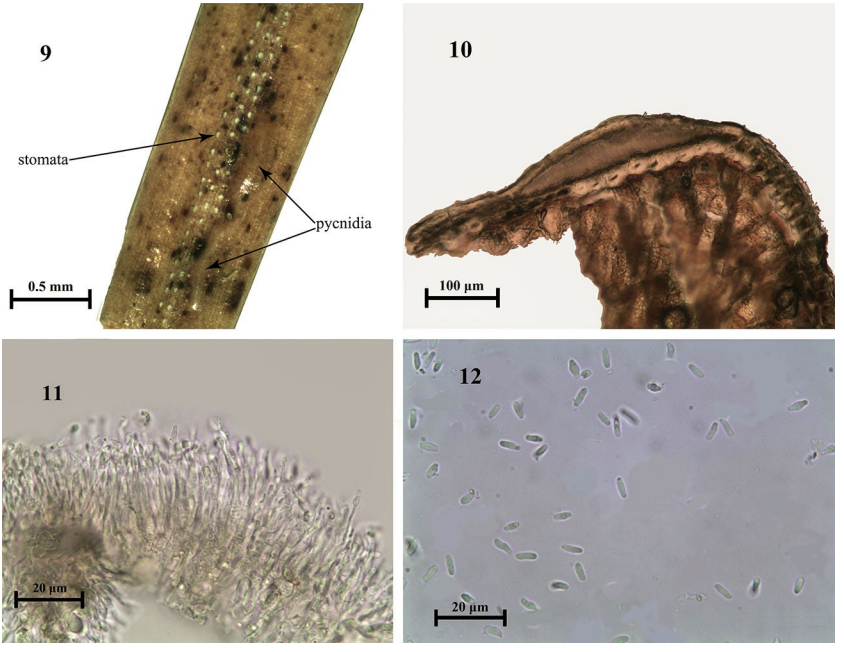
20 µm


8
20 µm



Figs. 9–12. *Lirula sichuanensis* (SAUF1606001) asexual stage on *Picea* needles. 9. Pycnidia observed under the dissecting microscope. 10. Pycnidium in vertical section. 11. Sporogenous tissue. 12. Conidia.

Additional specimens examined—**CHINA, Sichuan Province, Pingwu County,** Yangdonghe Plantation, alt. 2822 m, on *Picea likiangensis* var. *rubescens*, 13 June 2016, X.L. Xu & C.L. Yang 160613 (SAUF1606001; GenBank KY399846); **Ganzi Autonomous Prefecture, Luhuo County**, alt. 3240 m, on *Picea likiangensis* var. *rubescens*, 30 July 2014, X.L. Xu & C.L. Yang 140730 (SAUF1407001).

Discussion

Few gene sequences of *Lirula* are available in GenBank. ITS rDNA sequence analysis clustered *L. sichuanensis* in an independent lineage with *L. macrospora* and *L. yunnanensis* on a branch with 70% bootstrap support apart from *L. exigua* in a distant clade. *Lirula exigua* differs from *L. sichuanensis*, *L. macrospora*, and *L. yunnanensis* (Darker 1932, Fan et al. 2012) in its paraphyses not swollen at tips, narrower asci, shorter ascospores, and conidiomata that are scattered and not in continuous rows. Additionally, *L. exigua* has been observed in needles on dead twigs; the areas of needles with ascomata are whitened and fragile; and the ascomal color and shape are somewhat different (Fan et al. 2012).

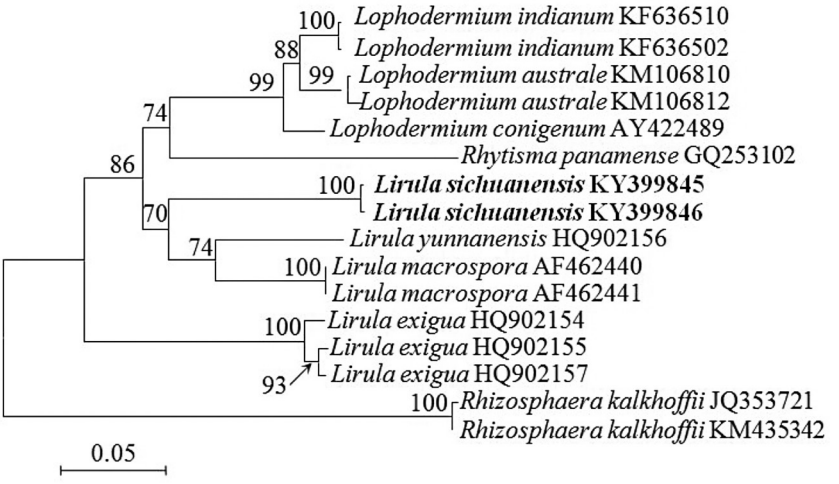


Fig. 13. Neighbor-joining phylogeny generated from partial ITS sequence analysis of *Lirula sichuanensis* and related species, using *Rhizosphaera kalkhoffii* as outgroup. Bootstrap values >80% from 1000 replications are shown on the respective branches.

Consequently, *L. exigua* needs further taxonomic study (including comparison with the type species, *L. nervisequa*) to establish whether it belongs in *Lirula*.

Lirula sichuanensis differs from other species in its distinctive paraphyses, which are longer and thicker, multi-septate, usually curved, and not to only slightly swollen at the tips (often branched near the middle or the apex) and thicker gelatinous sheath. In addition, the ascospores and gelatinous sheath are thicker, and the conidia are broader. The new species shares a similar habit with *L. brevispora* and *L. macrospora* on the needles of *Picea* spp. (Darker 1932, Ziller 1969) but differs from them by its longer thicker branched paraphyses, conspicuously larger or wider ascospores and gelatinous sheath, and inconspicuous basal stroma. *Lirula macrospora* differs in its smaller (100–132 × 14–16 μm) clavate-cylindric asci, much smaller (56–68 × 2.5–3 μm) clavate ascospores, unbranched paraphyses with swollen tips, irregularly scattered or sometimes 2–3 coalescent conidiomata that do not form two continuous or interrupted rows on needles (Darker 1932), much smaller (2.5–4.5 × 1.2–1.5 μm) conidia, and sometimes producing trichogynes (Lin et al. 2012). Ascal shape and size of *L. sichuanensis* is similar to that of *L. yunnanensis*, which differs by its bluish grey to bluish black ascomata, its narrower ascospores often

covered by gelatinous caps and a thinner gelatinous sheath, and its bigger, deeper, pale yellowish brown, epiphyllous, nervisequious pycnidia (Fan et al. 2012).

Lirula sichuanensis causes serious needle blight of *Picea likiangensis* var. *rubescens* in planted forests in Sichuan. It usually infects 1-year-old needles of 20- to 30-year-old trees from May to July and in the following March to May produces ascomata on 2-year-old needles. According to our survey, this disease spreads quickly in planted spruce forests. An overall investigation is needed to understand the scope and area affected by the disease, and this pathogen should be controlled at ecological security level by forestry measures or drug treatment.

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